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Disturbance of Plasma and Platelet Thrombospondin Levels in Sickle Cell Disease

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Thrombospondin (TSP), a large protein found in platelet α-granules (as TSP-1), mediates adhesion of sickle reticulocytes to cultured vascular endothelium. To further explore the physiologic relevance of this observation, we have measured plasma TSP levels and platelet TSP-1 content in subjects with sickle cell disease. Plasma TSP levels were similar for normal controls (mean 491 ng/ml, range 331–723) and steady-state HbSS patients (mean 536, range 333–1107) and were significantly ($P = 0.012$) but variably elevated for HbSS patients presenting with acute painful crisis (mean 868, range 442–2780). Some of these elevated plasma TSP levels reached those previously observed to support maximal red cell adhesion to endothelium in vitro. Compared to normals, both steady-state and in-crisis HbSS patients had significantly ($P < 0.001$) depressed platelet TSP-1 content (82.6 ± 11.9, 47.1 ± 16.0 and 45.9 ± 20.7 ng/10^6 platelets, respectively, mean ±SD). HbSC disease patients, all examined during steady state, had low-normal plasma levels of TSP and either normal or depressed platelet TSP-1 content. Serial observations on three sickle cell anemia subjects indicated a probable relationship between platelet TSP-1 release, elevated plasma TSP levels, and acute vasoocclusive episodes. These results suggest a state of ongoing release and depletion of TSP-1 from activated platelets in patients with sickle cell disease.

Key words: thrombospondin, platelets, sickle cell anemia

INTRODUCTION

Adhesion of sickle erythrocytes (RBCs) to vascular endothelium is believed to be an initiating factor for the development of sickle vasoocclusion [1,2]. A number of adhesogenic plasma proteins have been implicated as mediators of this event, including von Willebrand Factor, fibrinogen, and thrombospondin (TSP) [2]. Their participation raises the possibility that fluctuations of their plasma levels due to physiological stresses and concurrent illnesses would be of potential pathogenetic relevance. Recently it was suggested that adhesion mediated by TSP may be a major initiating factor in the pathogenesis of vasoocclusion in sickle cell disease [2–4].

TSP is a large, trimeric, modular protein having various domains implicated in cell binding [5–7]. Several members of the TSP gene family have been identified [8,9], but most data pertain to TSP-1, the form of TSP found in human platelets. Potential physiologic roles of this protein include participation in extracellular matrix functions [6], platelet aggregation [10,11], adhesion of hematopoietic progenitors [12], modulation of fibrinolysis [13], and inhibition of angiogenesis [14]. TSP is produced by a variety of cultured cells, including monocytes/macrophages, megakaryocytes, endothelial cells, smooth muscle cells, fibroblasts, glial cells, and various tumor cells. Significantly, TSP-1 is released from platelet α-granules during platelet activation and secretion [6,10,11].

The present studies were performed to determine whether TSP levels are abnormal in patients with sickle cell disease. Results indicate that this is the case and suggest that this is a consequence of in vivo platelet activation.

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METHODS

Blood Collection and Handling

Fresh blood was obtained from volunteer normal donors or from subjects with sickle cell anemia (HbSS) or hemoglobin (Hb) SC disease. Informed consent was given in all cases. The sickle subjects were studied when they first presented with acute painful crisis and/or when in their "steady state", at least one month remote from any acute clinical event. Sickle patients with acute clinical presentations other than painful crisis were excluded.

Blood was drawn into an anticoagulant mix comprised of citrate supplemented with adenosine, theophylline, and dipyridamole (Diatube H; Diagnostica Stago, France). In some cases, we further supplemented this anticoagulant mix with PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone), a high-affinity inhibitor of thrombin-mediated platelet activation [15], sufficient to yield 1 μM after dilution in blood. In most cases blood was obtained from a free-flowing, clean venipuncture using a large needle after release of the tourniquet. An occasional sample was obtained by indwelling venous access; these samples showed no tendency to yield different results from those obtained using our standard procedure.

Resulting blood samples were immediately centrifuged at room temperature at slow speed to obtain platelet-rich plasma (PRP), which was then centrifuged at 18,000*g* at 6°C for 15 min. Using our anticoagulant mix, we found lower TSP concentrations in platelet-poor plasma (PPP) prepared in this manner than at room temperature, a finding consistent with earlier studies [16]. The resulting PPP was saved by freezing. To make serum, blood was drawn into standard serum tubes without anticoagulant and was incubated for 2 hr at 37°C, after which serum separated from clot was saved by freezing. Samples were shipped for TSP assay frozen on dry ice.

TSP Content

TSP content of PPP, expressed in ng/ml, was determined using an enzyme-linked immunosorbent assay (ELISA)-based test that employs a monoclonal antibody (MAb) raised against purified TSP-1, as previously described [17]. Releasable platelet TSP-1 content, expressed in ng per 10^6 platelets, was calculated from the TSP content measured in serum and the platelet count determined at the time of blood sampling. Platelet counts were measured using a Coulter STKS automated counter. Microvesicular red cell fragments have been demonstrated previously in sickle blood [18]. However, the mean volume of these microvesicles, based on previous reports of their diameter [18,19], is estimated to be 0.05–0.1 fl, well below the minimum platelet volume of 2 fl detected by the automated counter in our study.

We chose the above method of quantifying platelet TSP-1 content rather than assay of purified washed platelets in order to avoid potential artifact from inadvertent, selective isolation of different platelet populations from normal and sickle cell individuals. Moreover, this method affords the advantage of ensuring that depressed values for platelet TSP content cannot be explained by artifactual loss due to platelet release during phlebotomy or subsequent sample handling. In control experiments, we verified that TSP concentration of serum prepared by our method was within 10% of the total TSP content of PRP. This justifies using releasable TSP-1 as a measure of platelet TSP-1 content.

Splenic Function

Fresh blood samples were added to an equal volume of 0.1 M cacodylate buffer (pH 7.4) containing 1% glutaraldehyde. These were examined microscopically using Nomarski optics to assess erythrocyte "pit count" as an indicator of splenic function [20]. We used values of >20% and >3.5% pitted RBC as the indicator of functional asplenia in HbSC [21] and HbSS [20] patients, respectively.

Statistical Analysis

Data were analyzed by Student's *t*-test. For plasma TSP levels, the data were log-transformed before analysis, which is appropriate because plasma TSP level can range from zero on the low side to perhaps an 100-fold increase on the high side; moreover, the actual range of TSP levels we found for sickle patients seems to confirm a log-normal distribution for this parameter (see below). Analysis of the same data without prior log-transformation did not eliminate the statistical significance of the data reported here.

RESULTS

Plasma TSP Level

The average level of TSP in PPP was similar for normals and for either HbSS or HbSC patients in their steady state, remote from an acute clinical event (Fig. 1). However, for HbSS patients presenting with acute painful episodes, plasma TSP levels were significantly elevated compared to the steady-state HbSS patients ($P = 0.012$). Actual values for means and standard deviations are presented in the legend to Figure 1. Of the 26 plasma TSP values obtained on the entire group of HbSS patients (regardless of clinical status), 38% were found to be above the range of the normals we studied. Additionally, in one HbSS patient we followed plasma TSP levels longitudinally and found a high degree of variability (Fig. 2). Notably, each in-crisis value is higher than the following steady-state value.

An interesting, but unexplained finding is that the

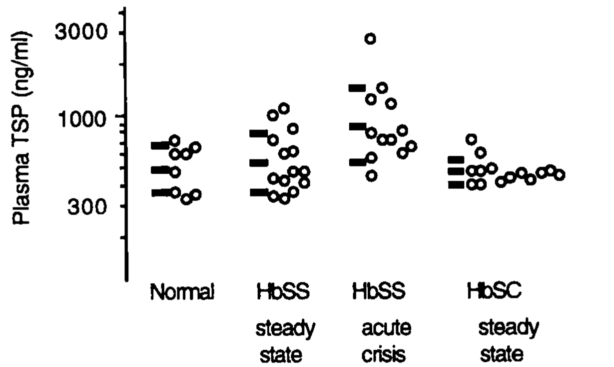

Fig. 1. Plasma TSP levels in sickle cell disease. Similar plasma TSP levels are found for normal controls (mean 491 ng/ml; values at ±1 SD = 358 and 673; n = 8), steady-state HbSC patients (mean 469; ±1 SD = 397 and 553; n = 14), and steady-state HbSS patients (mean 536; ±1 SD = 360 and 798; 14 measurements on 9 patients). However, plasma TSP level for in-crisis HbSS patients (mean 868; ±1 SD = 527 and 1,419; 12 measurements on 8 patients) is significantly elevated compared to the steady state HbSS patients ($P = 0.012$). Bars indicate mean and ±1 SD for each group.

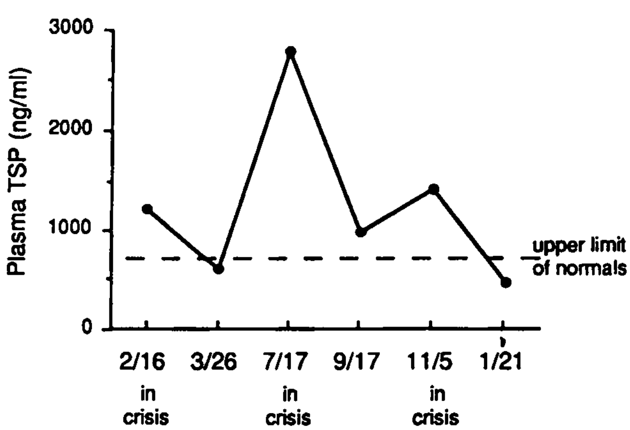

Fig. 2. Longitudinal measurement of plasma TSP level in an HbSS subject. Plasma TSP level was measured repeatedly when this patient presented with acute painful crises and at subsequent routine outpatient visits when in steady state.

plasma TSP values for the steady state HbSC disease subjects tended to be somewhat lower and to show much less interindividual variability than those obtained from the steady-state HbSS patients (Fig. 1). For the log-transformed data, the standard deviation for the HbSC subjects was only 0.072, while the data for normals, steady-state HbSS patients, and acute-crisis HbSS subjects were 0.137, 0.173, and 0.215, respectively.

In several experiments we examined the effect of adding PPACK to our standard anticoagulant mix. The plasma TSP level with PPACK was 94.9 ± 15.1 percent of that without PPACK, indicating that the addition of PPACK

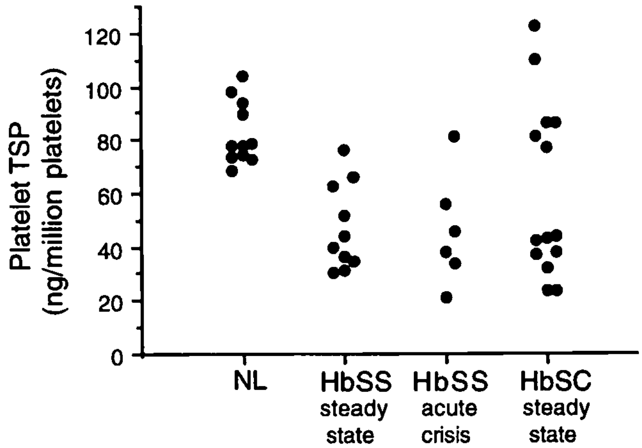

Fig. 3. Platelet TSP content in sickle cell disease. Platelet TSP content, inferred from TSP-1 release into serum (expressed in ng per 10^6 platelets), was significantly ($P < 0.001$) depressed for both steady-state HbSS subjects (47.1 ± 16.0, mean ±SD; 10 measurements on 9 patients) and for in-crisis HbSS subjects (45.9 ± 20.7; n = 6), compared to normal controls (82.6 ± 11.9; n = 11). A wide range of platelet TSP content was observed for steady-state HbSC patients (60.5 ± 32.4; n = 14; $P = 0.033$, compared to normal controls).

conferred no additional benefit over the basic anticoagulant mix.

Platelet TSP-1 Content

Compared to normal controls, both steady-state and in-crisis HbSS patients manifested significantly ($P < 0.001$) depressed platelet TSP-1 content, as calculated from TSP-1 released during blood coagulation in vitro (Fig. 3). Values for the means and standard deviations are presented in the legend to Figure 3. By contrast, HbSC patients, all of whom were examined in steady state, exhibited either normal or depressed platelet TSP contents ($P = 0.033$ versus normals). Indeed, inspection of Figure 3 suggests that all three sickle disease groups perhaps include some individuals that have normal platelet TSP-1 content: 1 of 6 of the acute-crisis HbSS patients, perhaps 3 of the 10 steady-state HbSS patients, and 6 of the 14 HbSC patients. The platelet TSP-1 content we observed for our normal donors is equivalent to that previously reported [22].

Correlations Between Parameters

We considered that elevated platelet counts in the sickle cell disease subjects [23] might contribute to elevated plasma TSP levels. As expected, platelet counts tended to be higher in patients with HbSS (396 ± 171, mean ±SD, $\times 10^9$/L) and HbSC (439 ± 294), than in normal donors (327 ± 81). However, we identified no significant

TABLE I. Paired In-Crisis and Steady-State Platelet/Plasma TSP Values in HbSS Subjects

HbSS donor	Platelet count ($\times 10^9$/L)	Plasma TSP level (ng/ml)	Platelet TSP-1 content (ng/10^6 platelets)
1 Steady state	240	333	75.8
Acute crisis	273	714	33.4
2 Steady state	340	989	65.6
Acute crisis	306	2780	38.2
3 Steady state	325	621	62.2
Acute crisis	216	559	56.0

correlation between blood platelet count and plasma TSP level for all subjects pooled or for any of the three individual genotypes analysed separately (data not shown), consistent with the lack of such correlation observed earlier for HbAA donors [22]. Nor was there any correlation between plasma TSP level and platelet TSP-1 content (data not shown).

We were able to obtain paired acute-crisis and steady-state samples for both plasma TSP and platelet TSP-1 on three HbSS patients (Table I). One of these patients showed no change in either parameter with change in clinical status. By contrast, the other two patients were observed to have lower platelet TSP-1 content at presentation with acute crisis than during steady state, and in both cases an inverse relationship between plasma TSP level and platelet TSP-1 content is apparent despite the fact that platelet count did not change significantly. This variability in sickle platelet TSP-1 content cannot simply be an artifact of measurement variability, since we established that platelet TSP-1 content for normal donors examined on a second occasion differed trivially from that on the first occasion (by only 5.6% ± 5.2%; n = 6 paired observations).

Blood smears were checked visually for the presence of platelet clumps or unusually large platelets, and no apparent correlation was noted between these features and plasma or platelet TSP levels.

Relationship to Splenic Function

We considered that TSP levels might be influenced by status of splenic function, which we assessed by performing RBC "pit counts." Neither plasma TSP level nor blood platelet count was significantly different for patients with or without splenic hypofunction (data not shown). By contrast, although platelet TSP-1 content clearly can be temporally variable (Table I), we nevertheless identified a tendency for it to be lower among patients with splenic hypofunction (45.3 ± 14.7 ng TSP/10^6 platelets, mean ±SD, n = 10) than among patients with normal splenic function (73.6 ± 35.4, n = 8, $P = 0.035$).

DISCUSSION

We have documented that plasma TSP levels tend to be elevated in HbSS patients presenting with acute painful crisis, compared to either normal controls or to HbSS subjects in steady state, remote from an acute clinical event. Repeated measurements on two HbSS patients further suggest a relationship between plasma TSP level and the acute vasoocclusive episode. One caveat is that for the present study, we obtained samples at presentation of acute crisis; it remains to be seen if a rise in plasma TSP level actually precedes crisis. Also, this clearly is not universally true since even some steady-state HbSS patients had elevated plasma TSP levels and since some HbSS patients in acute crisis did not. To some extent, this variability may derive from the ambiguity of definition of "steady state," which is a purely clinical judgment that may or may not reflect important biochemical differences [24]. Overall, 38% of the plasma TSP levels we obtained on the HbSS patients (regardless of clinical status) were elevated above the range we found for the normal subjects.

Some potential explanations for elevation of plasma TSP levels in sickle cell disease subjects can be excluded by available data, while others remain tenable. First, the lack of correlation between platelet count and plasma TSP level argues that elevated plasma TSP levels are not simply related to the higher number of circulating platelets manifested by some sickle cell disease subjects. Second, it is reported that plasma TSP levels tend to be elevated in HbAA donors who have been surgically splenectomized [25]. Thus, the "autosplenectomy" state of sickle patients might contribute to the elevated steady state TSP levels for some subjects. Against this, however, is the fact that plasma TSP levels for sickle cell disease patients clearly were not significantly increased for those with splenic hypofunction as defined by "pit" counts. Third, plasma TSP levels also tend to be elevated in HbAA subjects with chronic liver disease [25], so this might contribute to our finding of elevated levels in some steady-state HbSS subjects. Fourth, it is believed that nonplatelet sources do make some contribution to plasma TSP levels [22], so we cannot exclude a contribution of enhanced endothelial release of TSP in response to local hypoxia, for example. We hope to identify the specific type of TSP in sickle plasma in future studies. Finally, the theoretical possibility that platelet TSP-1 content varies as a function of platelet age and somehow thus contributes to the relationships observed here seems unlikely, given the fairly uniform TSP-1 content reported for platelets in patients with various types of thrombocytopenia [22]. The age of platelets in sickle cell disease patients is unclear, as platelet survival has been reported to be both shortened and lengthened in these patients [23].

Rather than the above possibilities, we believe that

elevated plasma TSP in HbSS subjects derives from in vivo platelet activation resulting in release of TSP-1 from platelet α-granules. Our observation of diminished platelet TSP-1 content in these patients is consistent with this hypothesis. Furthermore, serial studies in two HbSS subjects indicated a reciprocal relationship between platelet TSP-1 content and plasma TSP level. In the body of older literature suggesting that sickle cell disease is characterized by in vivo platelet activation, the major evidence for this has been demonstration of elevated plasma levels of platelet factor 4 and/or beta-thromboglobulin [23]. Our studies of both plasma and platelet TSP levels extend these observations. However, additional evidence of ongoing platelet activation in these patients would be desirable. To date, only preliminary reports have appeared describing abnormal expression of activation-specific antigens on the surface of platelets from patients with sickle cell disease [26,27]. The extent of in vivo platelet activation among sickle patients, as well as its temporal and causal relationship to clinical events, will require detailed, longitudinal studies of a much larger number of patients.

The above interpretation of our results must be reconciled with the observed discordance among HbSS patients, both in crisis and steady state, between fairly consistent depression of platelet TSP-1 contents and variably increased plasma TSP levels. Furthermore, 8 of 14 Hb SC patients, all in steady state, showed diminished platelet TSP-1 despite normal plasma TSP levels. This probably reflects the fact that plasma TSP has an extremely short half-life of 10–75 min [6], so that elevations of plasma TSP will be recorded only if abnormal α-granule release is occurring virtually at the moment of venipuncture. By contrast, platelet TSP-1 content will reflect the average status of circulating platelets, a longer-lived and time-averaged parameter. This would explain a lack of simple correlation between plasma TSP level and platelet TSP-1 content in our study subjects. Nevertheless, data from sickle patients on whom we were able to obtain paired platelet/plasma TSP values in both steady state and acute crisis do suggest a reciprocal relationship between platelet TSP-1 content and plasma TSP level.

Regarding this apparent instability of plasma TSP levels, it is intriguing that the blood levels of C-reactive protein are reported to be unstable in sickle cell disease patients, with the degree of instability being a predictor of the frequency of vasoocclusive involvement [28]. This could be directly relevant to platelet release of TSP-1, since C-reactive protein can stimulate tissue factor expression [29], which in turn would help explain the enhanced thrombin generation and ultimate platelet activation/release in sickle cell disease [23,30].

If platelet activation participates in acute vasoocclusion, it need not be by thrombosis per se. The plasma TSP levels of a number of the HbSS patients in this study reached or even exceeded the TSP concentration (1,000 ng/ml) we earlier found to promote maximal sickle red cell adhesion to endothelium in vitro [3]. Indeed, it is worth noting that these TSP levels we measured in venous blood might well be lower than levels in microcirculatory beds if the stimulus underlying their elevation is localized rather than widespread. Thus, the present data perhaps argue for a convergence of the influences of coagulopathy [23] and erythrocyte adhesion (i.e., as mediated by TSP) on the pathophysiology of vasoocclusion in sickle cell disease. Parenthetically, an additional, intriguing possibility relates to the ability of TSP to inhibit angiogenesis [14]. We speculate that the well-known higher risk of neovascularizing retinopathy among HbSC compared to HbSS patients could derive from the tendency of the former group to have lower plasma TSP levels.

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